iGEM Day 6

The team along with Professor Duffy attended a Synthetic Biology Conference at BU. Speakers included Patrick Boyle from Gingko Bio Works as well as Nobel laureate Jack Szostak, among numerous others. Jack Szostak was the first annual Charles Cantor lecturer, and his lecture was entitled “Building protocells to study the origins of life.” Also, graduate students from various colleges such as BU, Harvard, MIT, and Yale presented posters and the iGEM team members were able to view the posters and ask questions. After the conference, the team went for a delicious Italian dinner in the North End.

- Kayla and Mike checked the arsenic promoter/RBS transformation plate and no visible colonies were present.

iGEM Day 7

To solve the problem with the ribosome binding site, the group met to order an oligo consisting of a prefix, the ribosome binding site, and a suffix and the complement to this oligo. Primers for these sequences were also ordered. The primers, with codes iGEM 3-7, can be found in the **Primers** document.

- Alex purified the YPG DNA from last week using a Qiagen purification kit. A digest was performed using all 50 ul of purified product with Xba1 and Pst1. A 9C liquid culture for agglutination procedure testing was prepared and incubated at 37ºC overnight..

- Kayla and Mike performed a restriction digest and ligation of the chloramphenicol resistant ATF1 gene (banana odor BBa_J45014), the double terminator (BBa_B0015), and the ampicillin resistant linearized plasmid. A transformation was then performed using the ligation product. An ampicillin plate containing all of the competent cells in the transformation tube was incubated at 37ºC overnight.

- Corbyn and Shawna used the digestions that they had created the Friday before and then ligated both BCLA and the CAEV p-28 genes into the psB1C3 backbone following iGEM protocol of ligation. We also ligated both BCLA and CAEV p-28 together into the psB1C3 backbone.They also transformed these ligated plasmids into competent *E. coli*. These cells were plated on chloramphenicol plates. The plates were incubated at 37 degrees over night.

iGEM Day 8

Team: media day and agglutination

- Alex and Chloe prepared a 1:10 dilution of the previously grown 9C liquid culture and incubated at 37ºC for 4 hours. The resulting cultures were then washed with PBS twice and also concentrated by 40%. One culture was fixed with 100ºC in PBS on a heat block

for 1 hour, one was fixed with 100% methanol for 1 hour, one was fixed with 10% formamide in PBS for 1 hour, and one was fixed in 4% paraformaldehyde in PBS for 20 minutes. These were then washed 3 times in PBS, and brilliant blue was added. These were stored overnight at 4°C.

- Kayla and Mike removed the transformation plate from the warm room and found that only air bubbles were present on the plate. The digestion of the ATF1 gene and the double terminator were repeated and the volume was quadrupled so a gel could be run. The mini-prep of the double terminator wa snot run as a control, so it was not clear whether or not the double terminator DNA had been digested. Thus, new restriction digests were prepared according to the table below. The reaction tubes were then heated at 37°C for an hour and 80°C for 20 minutes.

Restriction Digest Volumes

	ATF1	DT
DNA Volume (uL)	2.63	3.85
dH2O Volume (uL)	30.37	29.15
NEB2 Volume (uL)	4	4
BSA Volume(uL)	1	1
EcoRI Volume (uL)	1	0
SpeI Volume (uL)	1	0
XbaI Volume (uL)	0	1
PstI Volume (uL)	0	1
Total Volume (uL)	40	40

- Corbyn and Shawna collected their plates from the warm room and found colonies on three of the plates. The results of the transformation plating can be seen below in the

table. The plates containing the bacteria that had used 2µl of the DNA insert of interest had more growth. The plates showing growth should have bacteria that contain the BCLA biobrick, the CAEV p-28 biobrick and a plasmid containing each of those two parts in the correct orientation. From each of these three plates 3 colonies were selected for liquid culture overnight at 37 degrees.

Results:

Plate With:	Number of Colonies:
2µl BCLA+ psB1C3 Backbone	4 single colonies and 2 streaks
6µl BCLA+ psB1C3 Backbone	0
2µl CAEV + psB1C3 Backbone	3
6µl CAEV + psB1C3 Backbone	0
3µl BCLA+ 1.5µl CAEV+ psB1C3 Backbone	10

<u>iGEM Day 9</u>

- Alex and Chloe prepared a test agglutination assay using a round bottom 96 well plate. The fixed bacterial samples were washed two more times. They then were analyzed under a microscope and found to have a very low bacterial concentration. The previously grown 9C culture was split into two tubes, where 1M IPTG* was added to one after 3 hours of incubation and grown for another hour. After incubation, the bacteria looked identical and both had an OD595 of 0.9. One culture was fixed with 100^{o}C in PBS on a heat block, while the other was fixed in 4% paraformaldehyde in PBS both for 20 minutes. These were then washed 3 times in PBS, and brilliant blue was added. These were stored overnight at 4^{o}C.
    - Calculations for 1M IPTG: MW = 238g/mol
        - 1 g = 0.0042 mol
        - 4.2 ml water + 1 g IPTG
    - Agglutination Assay: 96 well round bottom plate with 50 ml 5% BSA in each well being used. 2ul of GFP antibody and 50 ml of extra BSA was added to the first well of each different fixative. 50 ml was then transferred down each row as a serial dilution. 50 ml of the respective bacteria was then added to each respective well.

Final Plate Contents: Each well contained 50 ml 5% BSA and 50 ml bacteria

	1	2	3	4	5	6	7	8	9
A (Methanol Fixed)	Ctrl								
B (Methanol Fixed)	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
C (Heat Fixed)	Ctrl								
D (Heat Fixed)	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
E (Paraformaldehyde Fixed)	Ctrl								
F (Paraformaldeyde Fixed)	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
G (Formamyde Fixed)	Ctrl								
H (Formamyde Fixed)	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600

- Kayla and Mike ran a gel to see if the linearized plasmids, the double terminator, and the ATF1 gene were digested. 5 microliters of the hyperladder were added to the first well followed by 8 microliters of DNA mixed with 2 microliters of DNA in consecutive wells. All lanes had a band of similar length with the exception of the DNA from the double terminator mini-prep, indicating that the plasmids were linearized rather than circular and that the DNA had been properly digested. The gel can be seen below. (Lane 1-hyperladder, Lane 2-Linearized Kanamycin Plasmid Digest, Lane 3- Linearized Ampicillin Plasmid Digest, Lane 4- ATF1 digest, Lane 5- Double Terminator Digest, Lane 6- Double Terminator Mini-Prep)

Plasmid, ATF1, and Double Terminator Digest Gel from 6/12/14

Once it was clear that the DNA had been properly digested, eight different ligation reactions were prepared. The volumes of DNA, buffer, ligase, and water added to each tube can be found in the table below.

ATF1, Double Terminator, and Linearized Plasmid Ligation Volumes

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8
Amp Plasmid Volume (uL)	0	0	0	0	2	2	2	2
Kan Plasmid Volume (uL)	2	2	2	2	0	0	0	0
ATF1 Volume (uL)	1	1	3.5	2	1	1	3.5	2
DT Volume (uL)	1	3	3.5	2	1	3	3.5	2
dH2O Volume (uL)	5	3	0	11	5	3	0	11
Ligase Buffer Volume (uL)	1	1	1	2	1	1	1	2
Ligase Volume (uL)	0.5	0.5	0.5	1	0.5	0.5	0.5	1
Total Volume (uL)	10.5	10.5	10.5	20	10.5	10.5	10.5	20

The eight tubes were left at room temperature for an hour to allow the ligation reaction to occur. After the hour, 2 microliters of DNA from each tube were added to eight 50 microliter samples of competent *E. coli* cells to start the transformation reaction. The iGEM transformation protocol was followed up through the plating step. When it came

time to plate the transformed bacteria, all 250 microliters of each sample was poured onto a plate and spread using glass beads. The eight plates were placed in the warm room overnight to allow colonies to grow.

- Shawna and Corbyn collected their liquid cultures from the 37 degree shaker and prepared glycerol stocks which were then stored at minus 80; They then performed minipreps from the liquid cultures. Once the minipreps were completed they used a nano drop to investigate the amount of DNA in the miniprep samples. From these minipreps restriction digests were performed using EcoRI and Pst1 to determine if the BCLA, caev-p28, and the combination of the two were successfully ligated into to backbone. The resulting digest was run on a 10% agarose gel for ~40 minutes and the bands were examined under UV light. We were expecting to see a band at roughly 100 base pairs for the BCLA insert, roughly 500 base pairs for the CAEV-p28 insert, and about 600 base pairs for the combination of the two fragments. We unfortunately were unable to take a photo of the gel because the DigiDockIt System was not functioning properly. We decided to run the experiment first thing the next morning so we would be able to correctly record the results.

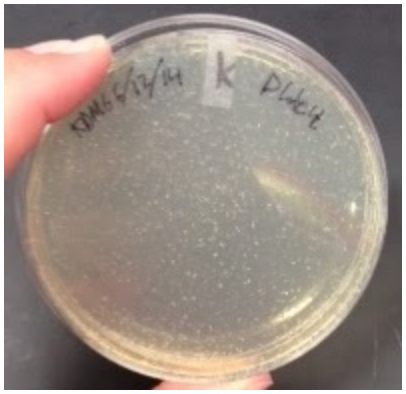
Day 10

- Alex and Chloe washed the two fixed bacterial samples twice with PBS. Another test agglutination assay was run using both samples as well as monoclonal and polyclonal GFP antibodies. The plate was covered and left overnight.

- Kayla and Mike resuspended the oligos that were ordered from IDT to 100uM in 10mM Tris buffer pH 8.0. 10uL of the forward and reverse RBS oligos were then annealed together in a boiling water bath until the water bath cooled to room temperature. Once the two strands were annealed, they were digested with EcoRI and SpeI. Additionally, the eight transformation plates in the warm room were checked for colonies. Two of the eight plates had visible colonies, so the transformation was finally completed successfully. The transformation with the greatest yield was that with the DNA from the ligation of 2uL of the kanamycin resistant linearized plasmid, 2uL of the ATF1 gene, 2uL of the double terminator, 2uL of buffer, 11 uL of water, and 1 uL of ligase. The successful transformation plates can be seen below.

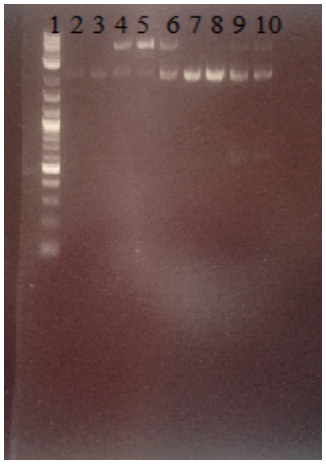
  Plate 1 Transformation Results

Plate 4 Transformation Results



The first experiment Corbyn and Shawna perfomed on Friday was once again running (in a 1.5% agarose) the EcoRI and SpeI digestion of the BCLA, CAEV-p28, and both in the backbone to check if the correct fragments were ligated into the backbone. The results we expected were to see a band at roughly 100 base pairs for the BCLA insert, roughly 500 base pairs for the CAEV-p28 insert, and about 600 base pairs for the combination of the two fragments. The resulting gel can be seen in the photo in this folder or below labeled **6/13/14 BCLA, CAEV,and BCLA+CAEV digest gel**. Each Lane is labeled and the corresponding sample is shown in the Table below.

Lane Number	Contents
1	Ladder
2,3,4	BCLA 1-3
5,6,7	CAEV 1-3
8,9,10	BCLA+CAEV 1-3



Lane 9 and 10 showed bands at roughly 600 basepairs, so we saved those samples as our 13C “BCLA+CAEV in the backbone” sample. However, the single digest and ligation of BCLA and CAEV into the backbone did not seem to work, so in order to test that we ran the original digestions of both BCLA and CAEV on a 1.5% agarose gel along with BCLA and CAEV undigested. The BCLA and CAEV were digested with E-S and X-P respectively. These gels were inconclusive therefore we intend to have samples sequenced. We believe the small bands that we expect from BCLA and CAEV may be too small to be clearly visible on the gel.

To end the day we performed a digestion of our BCLA-CAEV plasmid and ampicillin resistant plasmid backbone. We digested BCLA-CAEV with X-P and the ampicillin backbone with E-P. This was in preparation for ligation of the ribosome binding site and the BCLA-CAEV fragment into an ampicillin resistant backbone.